Journal of Medical Virology 60:200–204 (2000)

DNA Vaccination of Mice With Plasmid Expressing Human Papillomavirus 6 Major Capsid Protein L1 Elicits Type-Specific Antibodies Neutralizing Pseudovirions Constructed In Vitro

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Human papillomavirus 6 (HPV 6) causes benign condylomata. As a model for HPV vaccine development, we tested a HPV 6 DNA vaccine candidate, constructed by subcloning the major capsid protein (L1) gene into an expression plasmid having the cytomegalovirus promoter, for its immunogenicity in BALB/c mice. Three intracutaneous inoculations of the plasmid with a gene gun at 2-week intervals elicited anti-L1 serum antibodies. The antibodies were found to recognize highly type-specific, conformation-dependent epitopes, including those to neutralize pseudovirions capable of inducing β-galactosidase in infected monkey COS-1 cells. The data support the idea that immunization with DNA capable of expressing HPV L1 can be used as an HPV vaccine strategy for humans. ***J. Med. Virol. 60:200–204, 2000.***

KEY WORDS: HPV L1 protein; DNA immunization; HPV vaccine

INTRODUCTION

Human papillomaviruses (HPVs), consisting of more than 70 genotypes, constitute a group of DNA tumor viruses that cause a variety of epithelial lesions [zur Hausen, 1996]. Among more than 20 genital HPVs recorded to date, some (HPVs 16, 18, 31, 58, etc.) are known to cause cervical cancer and some (HPVs 6 and 11) to cause benign condylomata [Lorincz et al., 1991]. Cervical cancer could be prevented theoretically if prophylactic vaccines against HPVs are developed [zur Hausen, 1996]. Vaccine development, however, has been hampered by the lack of cell cultures to support the growth of HPVs in vitro, which are essential for production of virion vaccines and for assay of neutralization antibodies.

Because cell culture systems for propagation of HPVs are not available, surrogate systems for producing HPV capsids and for monitoring infection with HPVs have been devised. The major capsid protein L1 alone or L1 together with minor capsid protein L2, when expressed in eukaryotic cells by expression vectors, has been shown to self-assemble and to form HPV particles, L1-capsid or L1/L2-capsid, respectively [Hagensee et al., 1993; Kirnbauer et al., 1993; Rose et al., 1993; Volpers et al., 1994]. When L1-capsids are used as antigens for enzyme-linked immunosorbent assay (ELISA), both type-specific and cross-reactive antibodies against HPVs are detectable in sera from patients positive for HPV DNAs [Kirnbauer et al., 1994; Rose et al., 1994a, 1994b; Carter et al., 1995; Le Cann et al., 1995; Nonnenmacher et al., 1995; Matsumoto et al., 1997]. Type-specific neutralizing antibodies in anti-L1 sera raised by immunizing animals with L1-capsids have been detected using pseudovirions, which are L1/L2-capsids containing expression plasmids for reporter genes, to monitor HPV infection [Roden et al., 1996; Unckell et al., 1997; Kawana et al., 1998]. These recent findings have made the study of anti-HPV neutralizing antibodies possible, providing basic information for developing vaccine strategies against HPVs.

The direct inoculation of a host with a recombinant expression plasmid (DNA vaccination) can induce potent and long-lived humoral and cell-mediated immunity against many different pathogens including viruses [Giese, 1999; Whitton et al., 1999]. Thus, DNA vaccination is an attractive method to overcome difficulties associated with the lack of cell culture systems for growing HPV. In this study, therefore, we con-

Grant sponsor: Ministry of Health and Welfare for the Second-Term Comprehensive 10-Year Strategy for Cancer Control; Grant sponsor: Ministry of Education, Science, and Culture, Japan.

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Accepted 29 June 1999

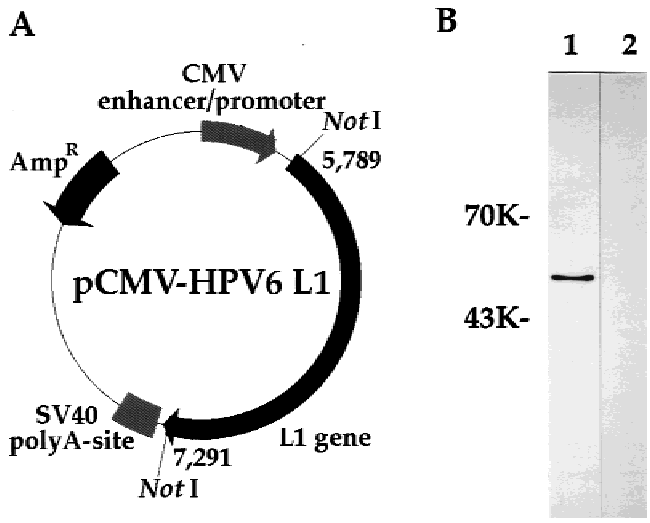

Fig. 1. **A:** Structure of pCMV-HPV 6L1, a HPV 6 DNA vaccine candidate. HPV 6 L1 gene, nucleotide number 5,789 to 7,291 [Schwarz et al., 1983], is placed under the control of CMV promoter in pCMV-0 vector. **B:** Expression of L1 protein in mouse NIH3T3 cells. Extract from cells transfected with pCMV-HPV6L1 were electrophoresed in 8% sodium dodecyl sulfate-polyacrylamide gels and analyzed by immunoblotting with the anti-HPV 16 L1 monoclonal antibody (PharMingen) which cross-reacts with HPV 6 L1[Matsumoto et al., 1997]. Extracts from cells transfected with pCMV-HPV6L1 (**lane 1**) and with pCMV-0 (**lane 2**).

structed a DNA vaccine candidate for HPV 6, as a model for HPV vaccine development, and examined its capability of inducing antibodies to neutralize pseudovirions, the in vitro constructed L1/L2-capsids containing expression plasmids for β-galactosidase. Sera from BALB/c mice vaccinated with an expression plasmid for HPV 6 L1 gene were found to contain anti-HPV 6 L1 antibodies recognizing type-specific, conformation-dependent epitopes including neutralization epitopes for pseudoinfection.

MATERIALS AND METHODS

DNA Vaccine Candidate, pCMV-HPV 6L1

An expression plasmid for HPV 6 L1 (pCMV-HPV 6L1) was constructed with pCMV-β (Clontech, Palo Alto, CA) as a backbone (pCMV-0) after the removal of the β-galactosidase (β-gal) gene. A DNA fragment encoding L1 protein, nucleotides 5,789 to 7,291, was amplified from cloned HPV 6b DNA [Schwarz et al., 1983] by polymerase chain reaction and inserted into pCMV-0 downstream of the cytomegalovirus (CMV) promoter/enhancer (Fig. 1A).

HPV Particles, L1-Capsids

L1-capsids of HPV 6b, 16, 18, and 58, used as antigens in this study, were produced by the use of recombinant baculoviruses and purified by CsCl density gradient centrifugation, as described previously [Matsumoto et al., 1997]. Denatured L1-capsids were prepared by boiling in phosphate-buffered saline (PBS) containing 1% sodium dodecyl sulfate (SDS) for 5 min. SDS was removed by repeated precipitation with cold acetone.

Immunoblotting Assay

Proteins in NIH3T3 cells transfected with pCMV-HPV 6L1 or mock were extracted in a buffer (Tris-HCl, pH 7.5, 1% SDS, and 2-mercaptoethanol), separated by 8% SDS-polyacrylamide gel electrophoresis, and blotted onto a Hybond-P PVDF membrane (Amersham Pharmacia Biotech Ltd., Sweden). The membrane was incubated with the anti-HPV 16 L1 monoclonal antibody (PharMingen, San Diego, CA), which has broad reactivities to HPV L1 proteins, including HPV 6 L1 [Matsumoto et al., 1997]. L1 protein on the membrane was visualized by the ECL Plus detection system (Amersham Pharmacia Biotech Ltd.) using the peroxidase-labeled goat anti-mouse IgG antibody (Cappel-Organinon Teknika, Westchester, PA).

Immunization of Mice

Gene gun immunization with the helium-powered Accell gene delivery system (Helios Gene Gun, BIO-RAD Laboratories, Hercules, CA) was used for inoculation of pCMV-HPV 6L1 or pCMV-0 extracted from *Escherichia coli* DH5a by alkaline-SDS method (QIAprep, Qiagen Gmbh, Germany) and purified by CsCl gradient centrifugation. Each shot contained 1.0 μg of the plasmid DNA precipitated onto 0.5 μg of 0.95 μm-diameter gold beads. Inoculation using a gene gun was given at 400 psi to the hair-removed skin on the abdomen of 6 BALB/c mice (1 shot per each mouse), and repeated three times at 2-week intervals. Sera were collected at 2 weeks after the final inoculation. To obtain mouse antisera against L1-capsids, 10 μg of HPV 6 L1-capsids were injected to a BALB/c mouse subcutaneously without adjuvant twice at interval of 4 weeks, and sera from 6 mice were collected at 2 weeks after the final immunization.

ELISA

Binding activities of antibodies to antigens were measured by ELISA using intact or denatured L1-capsids as antigens, as described previously [Matsumoto et al., 1997]. Wells of microtiter plates (Immuron II microtiter plate, Dynatech Laboratories, Inc., Chantilly, VA) that received 0.5 μg of the antigens were blocked with gelatin (0.2% in PBS). An aliquot of 45 μl of serum samples diluted in PBS (pH 7.0) containing 1% bovine serum albumin (BSA) was incubated in the well for 1 hr at room temperature. Wells were then washed nine times with PBS containing 0.05% Tween-20 and 0.05% NP-40. Then 50 μl of peroxidase-conjugated goat anti-mouse IgG (Cappel-Organon Teknika), anti-mouse IgG1 (Boehringer Mannheim Biochemicals, Germany), or anti-mouse IgG2a (Boehringer Mannheim Biochemicals) antiserum diluted at 1 to 2,000 in PBS containing 1% BSA prior to use was added to each well. Absorbency at 450 nm was measured after reaction with a mixture of 0.01% H_2O_2 and *o*-phenylenediamine (2 mg/ml) in 0.1 M citrate buffer

(pH 4.7) for 15 min at 20°C. Specific ELISA absorbency was calculated by subtracting the absorbency of mock samples measured for the wells that received gelatin only. Samples with specific absorbency of higher than 0.200 were considered to be positive.

Neutralization of Pseudovirions

Neutralizing activity was tested against in vitro reassembled HPV 6 pseudovirions containing an expression plasmid for β-galactosidase as described previously [Kawana et al., 1998]. The pseudovirions of 50 infectious units in 0.1 ml PBS (pH 6.8) were mixed with 0.1 ml of serial dilutions of the antisera in PBS (pH 6.8), incubated at 4°C for 1 hr, and then mixed with COS-1 cells (4×10^5), which had been dispersed with PBS containing EDTA (2.5 mM) and washed with Dulbecco's modified Eagle's medium before mixing, in 0.3 ml of PBS containing 0.01% BSA for 2 hr at 4°C with constant agitation. The cells were seeded into 6-well plates, grown for 36 hr, and fixed with glutaraldehyde. Cells expressing β-galactosidase were stained using X-gal as substrate (In Situ β-Galactosidase Staining Kit, Promega, Madison, WI). The number of blue cells was counted and the neutralizing activity was expressed as a reciprocal of maximum dilution of sera that reduced the number of blue cells to half of the sample treated with PBS containing BSA.

RESULTS

Ability of the DNA Vaccine Candidate to Produce HPV 6 L1 in Mouse Cells In Vitro

Expression of L1 from pCMV-HPV 6L1 in mouse NIH3T3 cells was shown by the immunoblotting (Fig. 1B). The extracts from cells transfected with pCMV-6L1 and the backbone plasmid (pCMV-0) were electrophoresed on an SDS-polyacrylamide gel and transferred to a nylon membrane. L1 was detected as a 56K protein in the lysate from cells transfected with pCMV-6L1. It was therefore concluded that pCMV-6L1 is capable of producing L1 in mouse cells.

DNA Immunization of Mice With pCMV-HPV 6L1

Like the HPV 6 particles (L1-capsids), the DNA vaccine candidate (pCMV-HPV 6L1), when administered with a gene gun, stimulated production of anti-L1 antibodies in BALB/c mice. The sera collected after the final immunization were examined for their binding activity to intact HPV 6 L1-capsids by ELISA. All of the serum samples from the mice immunized with either pCMV-6L1 or L1-capsids were found to bind at a dilution of 1:8,000 with the intact L1-capsids (Fig. 2A).

Characterization of Anti-L1 Antibodies

Immunization of mice with pCMV-HPV 6 L1 was found to elicit antibodies recognizing highly type-specific, conformation-dependent epitopes. The HPV 6 antibodies obtained with DNA immunization and with L1-capsids were examined for their cross-reactivity with other HPVs by ELISA with HPVs 16, 18, and 58

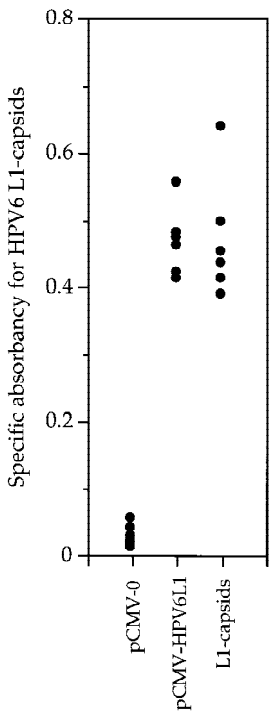

Fig. 2. Reactivity of serum from each mouse to L1-capsids of HPV 6. Specific absorbency in enzyme-linked immunosorbent assay (ELISA) measuring the serum from each mouse immunized with pCMV-0, with pCMV-HPV6L1, or with L1-capsids was plotted. Samples were diluted with phosphate-buffered saline at 1:8,000.

L1-capsids as antigens (Table I). The antibodies from DNA immunization bound exclusively with intact HPV 6 L1-capsids, but not with the denatured antigen. In contrast, the antibodies from capsid immunization showed low levels of cross-reactivity with HPVs 16 and 58 and bound with the denatured antigen.

DNA vaccination with pCMV-HPV 6 L1 was found to induce production of antibodies against HPV 6. The antisera were measured for their activity to inhibit the infection with HPV 6-pseudovirions, and the results were shown in Table II. Clearly, antibodies raised by DNA vaccination have neutralizing activities against pseudoinfection. The ratios of the neutralizing activity to the binding activity of the antisera obtained by the DNA vaccination was comparable to those of antisera obtained from mice immunized with L1-capsids.

DISCUSSION

Because HPVs cannot propagate and produce viral particles in cultured cells, DNA is an attractive candidate for a vaccine against HPVs. In this study, mouse serum antibodies induced by vaccination with expression plasmid for HPV 6 L1 were found to bind exclusively to HPV 6 L1-capsids and to neutralize the infectivity of HPV 6-pseudovirions.

Efficacy of prophylactic vaccines for papillomaviruses has been studied in detail using cottontail rabbit papillomavirus (CRPV) in rabbits. The immunization of rabbits with bacterially expressed L1 did not induce

TABLE I. Cross-Reactivities of Antibodies With L1-Capsids of HPV 16, 18, and 58*

Immunization of mice with	Reciprocal of dilution of antisera for HPV6	denatured HPV6	HPV16	HPV18	HPV58
Plasmid DNA	8,000	250<	250<	250<	250<
L1-capsids	16,000	1,000	2,000	250	2,000

*HPV, human papillomavirus.

TABLE II. Neutralization of Pseudoinfection

Immunization of mice with	Reciprocal of dilution of antisera for Binding with HPV 6 L1-capids[a]	Neutralizing of HPV 6 pseudoinfection
Preimmune	100>	100>
Plasmid DNA	8,000	1,000–3,300
L1-capsids	16,000	3,300–10,000

[a]HPV 6, human papillomavirus 6.

serum antibodies to neutralize CRPV and failed to protect the animals from experimental viral challenge [Lin et al., 1993]. Vaccination with L1-capsids induced high-titered neutralizing antibodies and was successful in protecting rabbits from infection [Breitburd et al., 1995]. The L1 gene DNA vaccine of CRPV has been constructed and administered to rabbits intracutaneously [Donnelly et al., 1996; Sundaram et al., 1997]. The vaccinated rabbits developed neutralizing antibodies and cellular proliferative responses and were protected from infection with the virus. Thus, it is likely that serum-neutralizing antibodies, which are induced only by the properly folded L1 in HPV particles or expressed endogenously, can prevent papillomavirus infection. Therefore, the results of this study suggest strongly that immunization of humans with the expression plasmid for HPV L1 may protect humans from infection with HPV in type-specific manner. Combination of expression plasmids for L1s of major high-risk HPVs will be a potential candidate for anti-multi-type vaccination.

The mouse antibodies induced by DNA vaccination were strictly type specific and conformation dependent, although the antibodies obtained by immunization of mice with L1-capsids showed low levels of cross-reactivity with HPV 16 and 58. Because endogenous antigen synthesis induced by DNA vaccine should mimic viral infection, human antibodies produced by infection with HPVs may be strictly type specific and conformation dependent. If this is the case, reactivity of an human serum sample to L1-capsids of multiple types of HPVs [Matsumoto et al., 1997] indicate that the donor was infected with the multiple types of HPVs in past. The characterization of antibodies induced by DNA vaccine in this study will provide a basis for analyzing seroepidemiological data more precisely.

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